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Cryopreservation of Bovine Oocyte Using Vitrification Solution and Cryotop Techniques

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ABSTRACT

Cryopreservation is used to preserve biological samples over an extended period at ultra-low temperatures. This process evolved into vitrification, a more advanced and superior technology in which fluids or water molecules form a glass-like structure without forming ice crystals. Unlike fresh cells, cryopreservation is reported to reduce oocyte viability and developmental competency. This study employed two vitrification techniques, vitrification solution (VS) and Cryotop, to investigate the meiotic resumption in bovine. Oocytes were extracted from cow ovaries collected from slaughterhouses in Banting and Shah Alam, Selangor, Malaysia. The oocytes were grouped (A, B, and B') based on cumulus morphology and matured *in vitro* in a culture dish (humidified 5% carbon dioxide incubator at 38.5°C) for 20 to 24 hr. Oocytes were vitrified after maturation using straws or aids of Cryotop sheets, then submerged in liquid nitrogen and stored for five days before defrosting for cryoprotectant elimination. By using Giemsa staining, the maturation state of fresh and vitrified bovine oocytes was evaluated through five parameters: zygotene, pachytene, diakinesis, metaphase I, and metaphase II. The maturation rate demonstrated only slight differences in the three groups of oocytes treated with VS (A: 44.79%; B: 30.97%; B': 20.70%) and Cryotop (A: 39.42%; B: 37.27%; B': 28.97%), which were significantly lower than fresh oocytes (A: 55.83%; B: 44.82%; B': 56.17%). Both VS and Cryotop methods were viable options for cryopreserving oocytes, but the Cryotop technique was more effective in increasing the meiotic competence of poor-quality oocytes.

Keywords: Bovine, cryopreservation, Cryotop, oocyte, vitrification

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INTRODUCTION

Oocyte cryopreservation has been actively investigated due to its powerful applications in livestock and human reproduction for more than three decades (Dhali et al., 2018). The ability to preserve the viability of reproductive cells and tissues on long-term freezing facilitates its application in animal and human reproduction (Vladimirov et al., 2019). Nevertheless, oocyte cryopreservation results in lower pregnancy and survival rates compared to freshly derived gametes, namely sperm, oocytes, and embryos (Basirat et al., 2016; Tharasanit & Thuwanut, 2021; Vining et al., 2021).

Despite the progress achieved in using various procedures such as *in vitro* maturation (IVM), *in vitro* fertilisation (IVF), and *in vitro* culture (IVC), there is still a need to improve the cryopreservation technique of oocytes in buffalo and bovine embryos produced under *in vitro* conditions (Sanches et al., 2019). Although modest reproductive technologies such as artificial insemination (AI), embryo transfer (ET), and IVF have been successfully applied in bovine and resulting in the birth of live offspring, *in vitro* produced (IVP), embryos are still not fully optimised in bovine and other domestic species (Aguila et al., 2020; Saunders & Parks, 1999). The success rate of oocyte cryopreservation is typically low compared to non-cryopreserved oocytes (Tharasanit & Thuwanut, 2021), although they survived both the freezing and thawing processes (Tao & Del Valle, 2008). Cryopreserved oocytes were reported to be more difficult than embryos (Dhali et al., 2018), as oocytes depicted high susceptibility to intracellular ice formation and low surface area to volume ratio (Tharasanit & Thuwanut, 2021). Several factors are responsible for the lower efficiency of *in vitro* production of embryos compared to *in vivo*, which could impact the development of bovine oocytes and their ability to develop into blastocysts (Abd El-Aziz et al., 2016). The impact of oocyte metabolic changes on lipid composition and cryo-tolerance are among the factors that occur in *in vitro* culture conditions compared to *in vivo* (Idrissi et al., 2021).

Cryopreservation procedures must, therefore, be improved to enhance the quality and yield of transferable bovine embryos. Cryopreservation often uses a vitrification solution technique to preserve living cells such as oocytes, zygotes, and blastocysts (Nagy et al., 2020). Such procedures ensure a consistent supply of oocytes and embryos for subsequent assisted reproduction applications such as *in vitro* embryo production (IVEP), embryo transfer (ET), stem cell production, and gene editing (Dhali et al., 2018). The vitrification technique can be defined as a cryopreservation process of biological samples in which a glass-like solution solidifies without forming ice crystals (Aljaser, 2022). A critical stage in a successful cryopreservation procedure is selecting and optimising freezing and thawing rates. This technique requires rapid cooling rates and concentrated cryoprotectant solutions to properly vitrify

the oocytes (Fathi et al., 2018; Kader et al., 2009; Reyes & Jaramillo, 2016).

Cryoprotectants, known as cryoprotective additive (CPA) solutions, are vital in freezing and thawing processes to prevent damage to samples. Samples may be damaged due to intracellular ice crystal formation with uncontrolled dehydration during freezing and 'osmotic shock' or swelling injury due to multiple flows between CPA solutions during solution warming (Sydykov et al., 2018). Warming the solution to 37°C and thawing in descending order of concentrated CPA to obtain gradual rehydration will reduce the osmotic shock in cryopreserved samples post-thawing (Kader et al., 2009; Whaley et al., 2021). The solution needs to be warmed to maintain the viability of oocytes and facilitate a faster recovery rate after sample preservation (Whaley et al., 2021). However, some CPAs, such as dimethyl sulfoxide (DMSO), are toxic to cells, especially at temperatures above 4°C (Tonev et al., 2020). Exposure to high levels of CPA during vitrification is hazardous to oocytes, resulting in parthenogenetic activation and zona hardening, compromising the subsequent development of vitrified/thawed oocytes (Fathi et al., 2018). Rapid freezing and thawing in a culture medium are important to reduce CPA toxicity (Jain & Paulson, 2006; Whaley et al., 2021). The toxicity of CPA can also be reduced by utilising two permeable CPAs instead of one to reduce or mitigate the toxic effect of a single CPA (Angel-Velez et al., 2021; Mahmoud et al., 2016; Nagy et al., 2009).

The common choices of CPAs in a vitrification procedure are sucrose and trehalose, which are non-permeable and can lessen the osmotic stress that develops throughout the process. In contrast, permeable CPAs such as glycerol, ethylene glycol (EG), DMSO, and methanol can penetrate the cells and replace intracellular water to generate osmotic stress (Angel-Velez et al., 2021; Do et al., 2016). New vitrification devices have also been developed to diminish the volume of the vitrification solution to accelerate the cooling process according to sample size (Bottrel et al., 2019; Fathi et al., 2018). In the latest development, a cryo referred to as the Cryotop technique is utilised in the cryopreservation process, which uses a minimum amount of vitrification solution (Bottrel et al., 2019; Cobo et al., 2008; Kuwayama, 2007). This method was initially employed in the cryopreservation of human oocytes, resulting in a 91% oocyte survival rate, followed by a blastocyst development rate of 50% (Kuwayama et al., 2005). Recent findings also demonstrated improved results when employing the Cryotop method in humans, with cleaved embryo survival rates and embryo live birth rates of 91.63 and 25.64%, respectively (Keshavarzi et al., 2022). These findings indicated that a combination of cryo-device and concentrated cryoprotectant led to higher survival rates of oocyte and pregnancy in humans, which can be applied to other developmental stages such as zygotes, cleavages, and blastocysts in bovine.

The Cryotop technique consists of thin polypropylene strips in which oocytes are loaded in an extremely small amount of the vitrification solution, a plastic handle, and a straw cover (Liu & Li, 2020). Cryotop accelerates the cooling rate through an open method, whereby samples are directly exposed to sterilised liquid nitrogen to avoid cryoinjury during chilling (Hochi, 2022; Liu & Li, 2020). The Cryotop device serves as a vitrification container for embryos and oocytes (Liu & Li, 2020), resulting in a high post-thawing survival rate (Kuwayama et al., 2005). The cryo-device has been reported to be more effective as the method entails a different strategy during the cooling process in which various carrier devices are used (Sripunya et al., 2010).

Presently, the government's restriction on slaughtering female animals, unless they are no longer productive, infertile, or diseased has contributed to the poor availability of cow ovaries in the local slaughterhouses. When there is abundant availability, the cryopreservation of bovine oocytes would be a suitable preservation measure to address the limited supply (Aljaser, 2022). Comparisons between two cryopreservation techniques, namely conventional and advanced cryo-device approaches, could offer improved guidelines for implementing these methods in bovine reproductive biotechnologies. Thus, this study aims to compare the Cryotop technique with the conventional cryopreservation method using a vitrification solution (VS). The meiotic resumption of the cryopreserved bovine oocytes was evaluated to determine the efficiency of the two techniques.

MATERIALS AND METHODS

Experimental Design

Oocytes with three different groups of cumulus cell layer (A, B, and B') were randomly assigned to the control group or unvitrified oocyte (Treatment 1), and two different freezing procedures: vitrification solution (Treatment 2) and Cryotop technique (Kitazato Supply Co., Japan) (Treatment 3). The meiotic status of the control group was determined immediately after IVM, whereas the two vitrification treatments were performed after IVM and freezing. After IVM, all oocytes were fixed, stained, and evaluated for their meiotic stages: zygotene, pachytene, diakinesis, metaphase I, and metaphase II.

Bovine Ovaries Collection

A total of 42 slaughtered native breed cattle from the slaughterhouse were sampled to collect ovaries. The cattle were slaughtered primarily owing to old age, illness, and sterility. Cattle ovaries were collected twice weekly from the local slaughterhouse in Banting and Shah Alam, Selangor, Malaysia and transported within two hours to the Gamete Laboratory, Livestock Science Research Centre, Malaysian Agricultural and Research Development Institute (MARDI) in Serdang. The ovaries were kept in phosphate-buffered saline (PBS, Sigma, USA) at 30 to 37°C during transportation from the slaughterhouse to the laboratory. Oocytes were obtained from the ovaries by slicing through the vesicular follicles using a sterile scalpel blade (Davachi et al., 2014), as shown in Figure 1 and were rinsed

with PBS supplemented with 10% foetal bovine serum (FBS, Hexcel-Berlin GmbH, Germany) before being subjected to IVM.

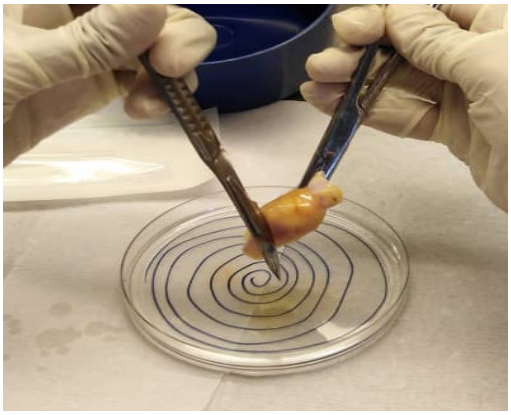

Figure 1. Oocytes were extracted from a bovine ovary using the slicing procedure

In vitro Maturation (IVM)

Cumulus-oocytes-complexes (COCs) were washed twice in tissue culture medium (TCM199, Sigma, USA) before being transferred and incubated in maturation droplets. The maturation droplet was prepared using TCM199 added with 0.1 M 17β-oestradiol (Sigma, USA) and 50 µm cysteamine solution (v/v) (Sigma, USA) and covered with mineral oil (Sigma, Canada). The COCs were loaded into the droplets according to the groups: Group A: packed and dense cumulus cell layer, Group B: less packed and less dense cumulus cell layer, and Group B': partial cumulus cell layer or almost naked (Bidin, 2005; Bidin et al., 2012) as in Figure 2. The oocytes were matured at 38.5°C in a humidified, 5% CO_2 incubator for 20 to 24 hr.

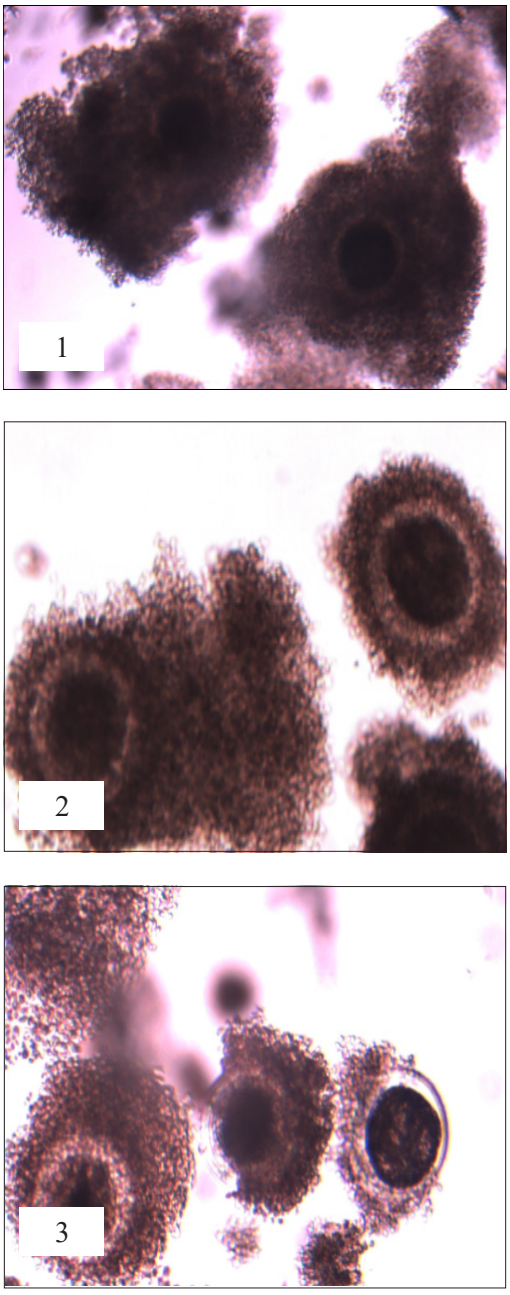

Figure 2. Oocytes used were differentiated based on the layer of cumulus cells around the oocytes (100× magnification)

Cryopreservation Technique

All media involved in the vitrification and thawing processes were kept at room temperature, except for the warming solution, which was kept at 37°C. Initially, matured oocytes were denuded and washed twice in TCM199 and added with 10% FBS for a few minutes. Two vitrification techniques, VS (Experiment 1) and Cryotop vitrification technique (Experiment 2), were used for this experiment. The VS solution for the conventional vitrification procedure was prepared according to Valdez et al. (1992), whereas the Cryotop device, together with the freezing and thawing media, was purchased from Kitazato Supply Co. (Japan).

Experiment 1: Conventional Vitrification Technique-freezing Technique. The vitrification process started by transferring the denuded matured oocytes into a 4-well plate containing 10% glycerol (Sigma, USA), VS1, VS2, and 1 M sucrose (Sigma, USA). Vitrification media was synthesised by preparing modified Dulbecco's Phosphate-Buffered Saline (m DPBS, Sigma, USA) upon mixing DPBS (Sigma, USA) with 15% FBS (Hexcel-Berlin GmbH, Germany). A 10% (v/v) glycerol (Sigma, USA) was prepared in m DPBS, whereas VS1 and VS2 were prepared using glycerol (Sigma, USA) and EG (Sigma, Germany) in m-DPBS in a ratio of 1:2:7 and 1:1:2, respectively. Denuded oocytes were incubated in 10% glycerol (Sigma, USA) for 5 min and later in the vitrification media twice for 6 min, 5 min in VS1 and immediately transferred to VS2 for another 1 min. In less than 1 min, m DPBS cleansed straw was loaded with 3 to 5 oocytes in VS2 with 1 M sucrose (Sigma, USA) at both ends of the straw, as shown in Figure 3. All straws were then submerged in a liquid nitrogen tank for storage.

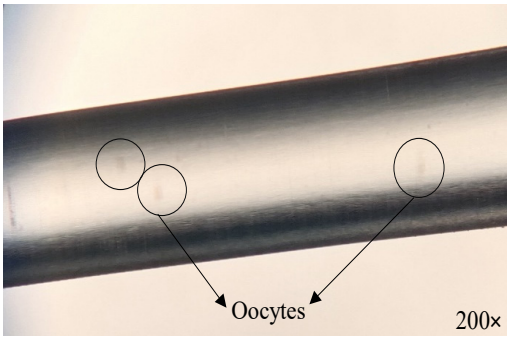

Figure 3. Matured oocytes were loaded into the conventional straw in VS2 solution for storage

Thawing Process. Thawing is required to remove the excess CPAs from the previous freezing technique. The EG (Sigma, Germany) and glycerol (Sigma, USA), which were used as CPAs in this study, were eliminated through a five-step procedure using thawing solutions from higher to lower concentrations of sucrose, starting from 1, 0.75, 0.50, 0.25, and 0.125 M in descending order. The sucrose concentration was prepared using m DPBS (Sigma, USA) mixed with sucrose (Sigma, USA). In the thawing process, oocytes were transferred into m DPBS containing sucrose with concentrations of 1, 0.75, 0.50, 0.25, and 0.125 M in sequence for 5 min each (Figure 4) before being transferred into m-DPBS. Oocytes were washed thrice in m-DPBS and transferred into TCM 199

(Sigma, USA) media supplemented with 17β-oestradiol (Sigma, USA). Finally, the oocytes were cultured in a humid, 5% CO_2 incubator at 38.5°C for 30 min before the fixing procedure.

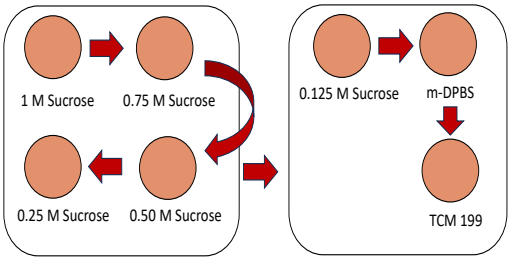

Figure 4. Vitrified oocytes were defrosted for 5 min each in descending concentrations of sucrose in the conventional straw thawing technique

Experiment 2: Cryotop Vitrification Technique-freezing Technique. Oocytes were vitrified using the Cryotop vitrification technique, consisting of a Cryotop device and commercial Cryotop media based on the manufacturer's instructions (Kitazato Supply Co., Japan). The latter comprises basic medium (BS), equilibration solution (ES), and vitrification solutions 1 (VS1) and 2 (VS2). A repro plate was prepared by adding 20 µl BS and 300 µl each of VS1 and VS2. The oocytes were transferred and left at the bottom of the 20 µl BS for several seconds. Oocytes were then equilibrated thrice by 1) adding a layer of 20 µl ES gently on the top of the BS and gently pushing the oocyte down the well and left for 3 min, followed by 2) 20 µl ES for 3 min before being supplemented with 3) 240 µl ES on top of the layer of the mixture from steps 1 and 2 for another 6 to 9 min. Oocytes were then exposed to the vitrification solution starting with VS1 for 30 s by sucking and releasing oocytes at various points using Pasteur pipettes and transferred into VS2 with a similar step to VS1 for another 30 s. A stereomicroscope was used to retrieve 3 to 5 oocytes and subsequently loaded into the Cryotop sheet (Figure 5) within less than 1 min of the oocyte's immersion in VS2. Almost all traces of BS, ES, and VS1 solutions from previous exposure covering the oocytes were eliminated, leaving only a small volume of less than 1 µl of VS2. Cryotop were plunged into liquid nitrogen after 1 min in a styrofoam container for storage before thawing.

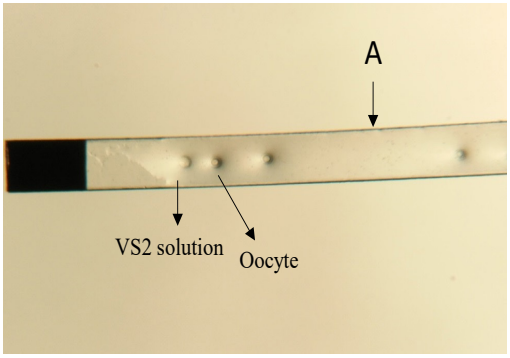

Figure 5. Oocytes were loaded onto Cryotop sheet (A) with a minimum amount of VS2 solution

Thawing of Vitrified Oocytes. The Cryotop sheet was removed from its protective cover, and the end of the polypropylene strip was immediately placed in the thawing solution (TS) for 1 min. Later, oocytes were carefully removed from the cryo-devices through gentle shaking and sucking in the oocytes using a 1 µl micropipette. After 1 min, the oocytes came off naturally from the Cryotop

sheet and transferred into the dilution solution (DS) using a 1 µl micropipette for 3 min. Oocytes were then incubated and washed twice in the first washing solution (WS1) for 5 min, followed by another minute in the second washing solution (WS2). The oocytes were then washed in a culture medium, placed in a culture dish containing the appropriate culture medium to prevent contamination, and further incubated to equilibrate for 1 hr (oocytes stabilise), as shown in Figure 6.

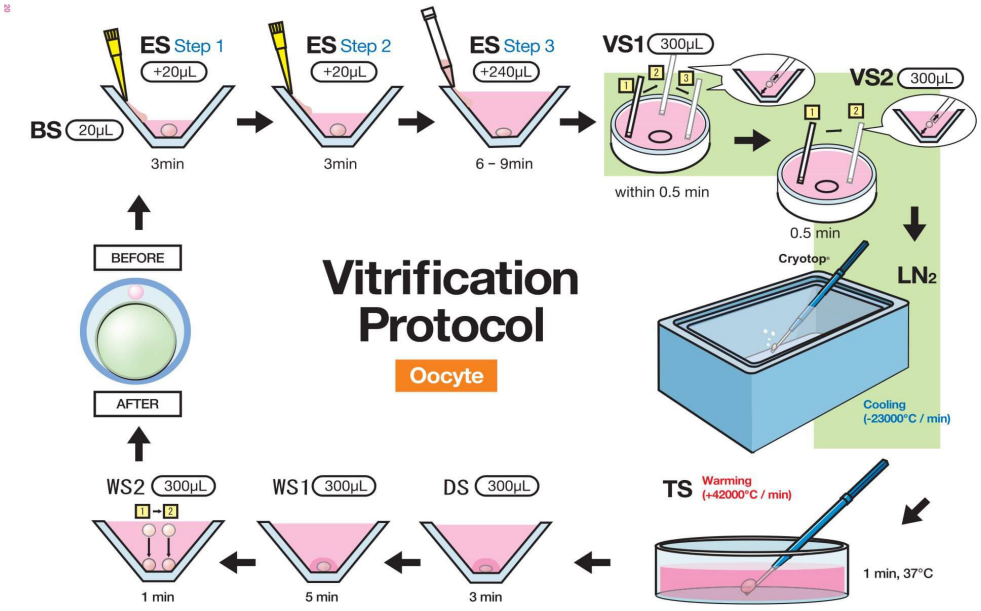

Figure 6. The procedure of freezing and thawing of bovine oocytes as per the manufacturer's instructions (Source: EXTOLSEED Oocyte and Sperm Bank, 2018)

Note. BS = Basic solution; ES = Equilibration solution; VS1 = Vitrification solution 1; VS2 = Vitrification solution 2; TS = Thawing solution; DS = Dilution solution; WS1 = Washing solution 1; WS2 = Washing solution 2

Fixing and Giemsa's Staining

Fixing and Giemsa's staining were performed as described by Bidin (2005). Both fresh and vitrified oocytes were retrieved using a stereomicroscope and treated with 1% hypotonic trisodium citrate solution (Sigma, Germany) individually for 3 min. Oocytes were immediately placed onto microscopic slides, and excess hypotonic trisodium citrate solution was removed before fixing with 1:1 (v/v) methanol (R&M Chemicals, United Kingdom) and acetic acid solution (Merck, Germany). The oocytes were then blow-dried. The microscopic slides were immersed in a Coplin jar containing the fixative solution of methanol and acetic acid (3:1) and kept overnight at 2°C. The slides were air-dried the next day and stained with 4% Giemsa solution (Sigma, Germany) for 3 min. After that, the slides were cleaned in xylene solution (Merck, Germany) for another 3 min and mounted with dibutyl

phthalate polystyrene xylene (DPX, Merck, Germany) before observation.

Evaluation of Meiotic Progression

The stages of nuclear maturation of stained oocytes were observed under a phase contrast microscope (Zeiss, Germany) at 100×, 200×, 400×, and 1,000× magnifications. The meiotic stages were categorised as zygotene, pachytene, diakinesis, first metaphase (MI), and second metaphase (MII). Oocytes arrested at MII were considered to have accomplished maturation *in vitro* (Figure 7), while germinal vesicle and MI were considered immature *in vitro*. An unidentified chromosome (OUC) is in a state where the chromosome is condensed or structurally abnormal and cannot be identified.

Data Analysis

The data on bovine oocytes' meiotic progression were analysed using Statistical Product and Service Solutions (SPSS, version 24). A one-way analysis of variance (ANOVA) with Duncan's multiple range comparison test was performed to assess the differences between the treatment means. The mean values were considered statistically significant at $p<0.05$.

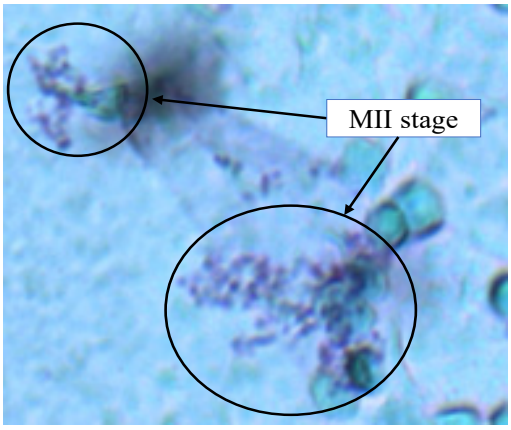

Figure 7. Stained metaphase II (MII) oocytes were observed under 400× magnification

RESULTS AND DISCUSSION

This study focused on the maturation rate of bovine oocytes as an indicator of meiotic competence and capability to resume meiosis at various stages, namely, zygotene, pachytene, diakinesis, metaphase I (MI), metaphase II (MII), and unidentified chromosomes under *in vitro* conditions. The meiotic resumption of vitrified bovine oocytes compared to the fresh oocytes as control is shown in Table 1. Significant differences ($p<0.05$) were observed in the zygotene, MI, MII, and unidentified chromosomes in the three treatments but not in the pachytene and diakinesis stages.

In terms of maturation rate, the mean percentage for MII recorded was significantly different ($p<0.05$) compared to the oocytes exposed to either vitrification or Cryotop treatments. The mean percentage of oocytes arrested at MII in the control group, Cryotop, and VS treatments were 52.27, 35.22, and 32.15%, respectively. Significant differences ($p<0.05$) were observed in the mean percentage of MI stage in control oocytes (1.54%) compared to the VS (9.57%) and Cryotop treatments (10.90%). Thus, in the present study, the maturation stage reflected that the meiotic competence of cryopreserved oocytes was lower than that of fresh oocytes. This result suggests that the cryopreserved oocytes

are probably susceptible to physical and chemical stress to resume their meiosis (Iussig et al., 2019); thus, the oocytes are less capable of surviving the cooling and warming procedures compared to normal fresh oocytes.

Table 1
The effects of conventional vitrification solution and Cryotop techniques on the mean percentage of meiotic stages in bovine oocytes

Treatment	Meiotic stages ± SEM						
	n	Zygotene	Pachytene	Diakinesis	MI	MII	OUC
Control	99	5.62 ± 2.87$^{a, x}$	8.18 ± 2.99$^{a, x}$	0.40 ± 0.40$^{a, x}$	1.54 ± 0.97$^{a, x}$	52.27 ± 6.52$^{b, y}$	8.18 ± 2.75$^{a, xy}$
Vitrification solution	115	5.29 ± 1.88$^{a, x}$	7.52 ± 2.72$^{ab, x}$	3.43 ± 1.98$^{a, x}$	9.57 ± 2.79$^{ab, y}$	32.15 ± 5.08$^{c, x}$	16.12 ± 3.98$^{b, y}$
Cryotop	95	16.67 ± 4.23$^{b, y}$	8.10 ± 2.50$^{ab, x}$	3.08 ± 1.49$^{a, x}$	10.90 ± 2.55$^{ab, y}$	35.22 ± 5.20$^{c, x}$	5.51 ± 2.09$^{a, x}$

Note.
$^{a, b, c}$ = Means within a row with different superscripts were significantly different at $p<0.05$;
$^{x, y}$ = Means within columns with different superscripts were significantly different at $p<0.05$;
n = Number of oocytes; Zy = Zygotene; Pa = Pachytene; Dia = Diakinesis; MI = Metaphase I;
MII = Metaphase II; OUC = Oocytes with unidentified chromosome
SEM = Standard error of the mean

In the zygotene stage, there were significant differences ($p<0.05$) in the mean percentage of control (5.62%) and VS (5.29%) treatments compared to Cryotop (16.67%), as shown in Table 1. Immature oocytes (GV stage) were more resistant using the Cryotop technique. A recent study reported that the DNA of the GV stage oocytes is more resistant to cryopreservation since it is relatively compact in the meiotic state and protected by the nuclear membrane (Peinado et al., 2022). Compared to the control at 8.18%, a significantly higher percentage of oocytes with unidentified chromosomes was observed in the VS-cryopreserved group compared to Cryotop-cryopreserved oocytes at 16.12 and 5.51%, respectively. This condition may result from the structural or molecular changes during the vitrification process using carriers or cryo-devices, thereby causing the microtubule's spindle to depolymerise and disorganise. These events would limit the viability and developmental potential of the oocytes (Mogas, 2018).

In addition, the conditions of oocyte donors in the present study in terms of breed, age, body scores, and parity numbers were unknown as the oocytes were harvested from slaughterhouse ovaries. It is a pertinent point as donor conditions affect the survival rate of oocytes (Tharasanit & Thuwanut, 2021). During the vitrification treatments, oocytes were exposed to various conditions, including chemical toxicity, osmotic shifts, and mechanical and thermal stresses, which may further reduce the oocytes' competence *in vitro*. As a result, the mean percentage

of vitrified oocytes with unidentified chromosomes increased compared to controls (Tharasanit & Thuwanut, 2021). Another reason might be technical errors while using the carriers to preserve the oocytes. Technical competence of personnel is very important in the vitrification techniques, especially during the handling and loading of oocytes, which must be accomplished within a short time. Moreover, it is difficult to visualise the oocytes in the highly concentrated solution, which could delay the vitrification process.

The mean percentage of various meiotic stages of A, B, and B' of bovine oocytes are shown in Table 2. In the control treatment, significant differences ($p<0.05$) were observed in oocytes reaching the MII stage compared to other stages in each group of oocytes. A higher mean percentage was observed in the MII stage compared to other meiotic stages, specifically in group B' followed by groups A and B at 56.17, 55.83, and 44.82%, respectively. However, no oocytes in groups B and B' were observed to reach the diakinesis stage in the control treatment.

Table 2
The effects of conventional vitrification solution and Cryotop techniques on the mean percentage of meiotic stages in three groups of bovine oocytes

Treatment	Group	Meiotic stages of oocytes ± SEM						
		n	Zygotene	Pachytene	Diakinesis	MI	MII	OUC
Control	A	34	9.17 ± 4.86$^{a, x}$	9.17 ± 4.36$^{a, x}$	1.19 ± 1.19$^{a, x}$	0.60 ± 0.60$^{a, x}$	55.83 ± 11.84$^{b, x}$	2.62 ± 1.79$^{a, x}$
	B	29	7.69 ± 7.12$^{a, x}$	10.62 ± 7.33$^{a, x}$	0.00 ± 0.00$^{a, x}$	1.65 ± 1.65$^{a, x}$	44.82 ± 11.99$^{b, x}$	6.65 ± 3.93$^{a, x}$
	B'	36	0.00 ± 0.00$^{a, x}$	4.76 ± 3.23$^{a, x}$	0.00 ± 0.00$^{a, x}$	2.38 ± 2.38$^{a, x}$	56.17 ± 10.59$^{b, x}$	15.26 ± 6.83$^{a, x}$
Vitrification solution	A	46	5.34 ± 3.04$^{a, x}$	9.26 ± 6.49$^{a, x}$	9.17 ± 5.69$^{a, x}$	9.31 ± 4.10$^{a, x}$	44.79 ± 9.49$^{b, x}$	11.02 ± 5.82$^{a, x}$
	B	34	6.94 ± 3.94$^{a, x}$	7.13 ± 3.49$^{a, x}$	1.11 ± 1.11$^{a, x}$	5.28 ± 3.14$^{a, x}$	30.97± 8.47$^{b, x}$	9.68 ± 3.71$^{a, x}$
	B'	35	3.57 ± 2.84$^{ab, x}$	6.17 ± 3.81$^{ab, x}$	0.00 ± 0.00$^{a, x}$	14.11 ± 6.61$^{abc, x}$	20.70 ± 7.93$^{bc, x}$	27.67 ± 9.42$^{c, x}$
Cryotop	A	35	13.78 ± 5.20$^{a, x}$	6.09 ± 3.96$^{a, x}$	0.00 ± 0.00$^{a, x}$	6.09 ± 2.78$^{a, x}$	39.42 ± 9.58$^{b, x}$	3.85 ± 2.60a$^{, x}$
	B	32	11.36 ± 5.45$^{a, x}$	12.18 ± 5.55$^{a, x}$	6.68 ± 3.55$^{a, x}$	15.20 ± 5.41$^{a, x}$	37.27 ± 7.65$^{b, x}$	1.92 ± 1.92$^{a, x}$
	B'	28	24.87 ± 10.24$^{bc, x}$	6.03 ± 3.27$^{ab, x}$	2.56± 2.56a$^{, x}$	11.41 ± 4.63$^{abc, x}$	28.97 ± 10.07$^{c, x}$	10.77 ± 5.25$^{abc, x}$

Note.
$^{a, b, c}$ = Means within a row with different superscripts were significantly different at $p<0.05$; ^{x}= Means within the column within a group with similar superscripts were not significantly different at $p>0.05$; n = Number of oocytes; MI = Metaphase I; MII = Metaphase II; OUC = Oocytes with unidentified chromosome
SEM = Standard error of the mean

In the VS treatment, groups A and B oocytes depicted significant differences (p<0.05) in MII compared to other meiotic stages. In group B' oocytes, a significant difference (p<0.05) was observed in unidentified chromosomes compared to other meiotic stages. Meanwhile, no significant difference at all meiotic stages was observed across the groups in the VS treatment. Although not significant, a higher mean percentage of oocytes in MII was observed in group A (44.79%), followed by groups B (30.97%) and B' (20.70%) oocytes. In the VS technique, the mean percentage of oocytes with unidentified chromosomes in groups A, B, and B' was 11.02, 9.68, and 27.67%, respectively. In contrast, no group B' oocytes reached the diakinesis stage.

Group B' oocytes had the highest mean percentage of unidentified chromosomes in comparison to groups A and B in the VS technique. It could be due to the warming and cooling steps in the vitrification process of the oocytes that were detrimental, thereby leading to cryodamage and affecting the oocytes' viability (Thrasanit & Thuwanut, 2021). In addition, the sensitivity of oocytes to cryopreservation varied depending on their meiotic stage (Rienzi et al., 2010). In this study, the oocytes' ability to enter the meiotic phase could be explained by maturation media containing serum and cysteamine to encourage oocyte growth (Bidin, 2005). The Cryotop had a similar trend as the control and VS techniques in which no significant difference was observed across the three groups of oocytes in all meiotic stages. However, a significantly (p<0.05) higher percentage of oocytes were observed in groups A and B oocytes reaching MII compared to other meiotic stages. The mean percentage recorded was 39.42, 37.27, and 28.97% for groups A, B, and B', respectively. For group B' oocytes, a significant difference (p<0.05) was observed in the diakinesis (2.56%) and MII (28.97%) stages compared to other stages. Oocytes in group B' were less likely to meiotically arrest in diakinesis, which might be attributed to poor cell-to-cell communication that lowers the cyclic adenosine monophosphate (cAMP) levels (Bidin, 2005).

The disconnection of the junctional competence in group B' oocytes could result from its characterisation, which is either a partial or almost complete lack of cumulus cells (Bidin, 2005). Although not significantly different, group B' oocytes had higher percentages of oocytes reaching zygotene (24.87%) and incidences of oocytes with unidentified chromosomes (10.77%). These might be due to insufficient cysteamine supplementation in the maturation media or the failure of the inferior quality of group B' oocytes to support full meiotic progression. The result might also stem from the cumulus cell dispersion component within the collection in group B', which results in meiotic arrest in this phase (Bidin, 2005). Cumulus cells are crucial in oocyte meiotic maturation and meiosis, which are necessary for ovulation, fertilisation, and subsequent early embryo development (Turathum et al., 2021). Therefore, understanding the involvement of oocyte grouping can be crucial in predicting

oocyte quality and subsequent embryonic development competence.

The percentage of meiotic resumption of fresh bovine oocytes (control), compared to conventional VS- and Cryotop-vitrified oocytes of three groups of oocytes, are presented in Table 3. A similar trend was observed, where A, B, and B' groups of oocytes of all treatments recorded the highest mean percentage of oocytes reaching MII. In group A oocytes, no significant difference was observed for all meiotic stages between the three treatments, although the mean percentage of oocytes reaching the MII stage was relatively higher in control (55.83%), VS (44.79%), and Cryotop (39.42%) groups. However, a significant difference ($p<0.05$) was observed in MII upon comparing the other meiotic stages within treatments in group A oocytes. In group B oocytes, significant differences ($p<0.05$) were observed in the diakinesis and MI stages of Cryotop treatment compared to other oocyte groups and treatments. Nevertheless, the diakinesis stage of group B oocyte was not detected in the control treatment.

Table 3
The mean percentage of meiotic stages in three groups of oocytes using conventional vitrification solution and Cryotop techniques

Group	Treatment	Stages						
		n	Zygotene	Pachytene	Diakinesis	MI	MII	OUC
A	Control	34	9.17 ± 4.86$^{a, x}$	9.17 ± 4.36$^{a, x}$	1.19 ± 1.19$^{a, x}$	0.60 ± 0.60$^{a, x}$	55.83 ± 11.84$^{b, x}$	2.62 ± 1.79$^{a, x}$
	Vitrification solution	46	5.34 ± 3.04$^{a, x}$	9.26 ± 6.49$^{a, x}$	9.17 ± 5.69$^{a, x}$	9.31 ± 4.10$^{a, x}$	44.79 ± 9.49$^{b, x}$	11.02 ± 5.82$^{a, x}$
	Cryotop	35	13.78 ± 5.20$^{a, x}$	6.09 ± 3.96$^{a, x}$	0.00± 0.00$^{a, x}$	6.09 ± 2.78$^{a, x}$	39.42 ± 9.58$^{b, x}$	3.85 ± 2.60$^{a, x}$
B	Control	29	7.69 ± 7.12$^{a, x}$	10.62 ± 7.33$^{a, x}$	0.00 ± 0.00$^{a, x}$	1.65 ± 1.65$^{a, x}$	44.82 ± 11.99$^{b, x}$	6.65 ± 3.93$^{a, x}$
	Vitrification solution	34	6.94 ± 3.94$^{a, x}$	7.13 ± 3.49$^{a, x}$	1.11 ± 1.11$^{a, x}$	5.28 ± 3.14$^{a, xy}$	30.97 ± 8.47$^{b, x}$	9.68 ± 3.71$^{a, x}$
	Cryotop	32	11.36 ± 5.45$^{a, x}$	12.18 ± 5.55$^{a, x}$	6.68 ± 3.55$^{a, y}$	15.20 ± 5.41$^{a, y}$	37.27 ± 7.65$^{b, x}$	1.92 ± 1.92$^{a, x}$
B'	Control	36	0.00 ± 0.00$^{a, x}$	4.76 ± 3.23$^{a, x}$	0.00 ± 0.00$^{a, x}$	2.38 ± 2.38$^{a, x}$	56.17 ± 10.59$^{b, y}$	15.26 ± 6.83$^{a, x}$
	Vitrification solution	35	3.57 ± 2.84$^{ab, x}$	6.17 ± 3.81$^{ab, x}$	0.00± 0.00$^{a, x}$	14.11 ± 6.61$^{abc, x}$	20.70 ± 7.93$^{bc, x}$	27.67 ± 9.42$^{c, x}$
	Cryotop	28	24.87 ± 10.24$^{bc, y}$	6.03 ± 3.27$^{ab, x}$	2.56 ± 2.56$^{a, x}$	11.41 ± 4.63$^{abc, x}$	28.97 ± 10.07$^{c, x}$	10.77 ± 5.25$^{abc, x}$

Note.
$^{a, b, c}$ = Means within a row with different superscripts were significantly different at $p<0.05$; $^{x, y}$ = Means within columns within a group with different superscripts were significantly different at $p<0.05$; n = Number of oocytes; MI = Metaphase I; MII = Metaphase II; OUC = Oocytes with unidentified chromosome

The phenotype of increased aneuploidy and lower developmental competence may be related to alterations in mitochondrial distribution at the level of cytoplasmic maturation (Rybska et al., 2018). In all treatments, a significant difference (p<0.05) was recorded in MII compared to other stages in group B oocytes. The highest MII that was achieved in group B oocytes was recorded in the control (44.82%), followed by Cryotop (37.27%) and VS (30.97%), respectively. These results indicated that the cryo-device, which only requires a minimum volume of solution for cryopreservation, could be less detrimental and thus sustain the structural integrity of oocytes (Cobo et al., 2008; Kuwayama, 2007). Group B' oocytes depicted statistically significant differences (p<0.05) between the three zygotene and MII stages treatments. In the MII stage, significant differences (p<0.05) were observed in control (56.17%) compared to Cryotop (28.97%) and VS (20.70%), the lowest being group B'.

It was not surprising that the survivability of fresh oocytes was higher compared to the vitrified oocytes. The sub-lethal damage caused the impaired meiotic progression of vitrified oocytes due to the cooling or warming procedures (Sripunya et al., 2010). The fresh oocytes had a higher percentage of meiosis rate since the detrimental effects of osmosis less impacted them through several equilibration and dilution and morphological injuries compared to vitrified oocytes (Sripunya et al., 2010). The structural and morphological damages were reported in vitrified-warmed oocytes, as stated by Amidi et al. (2018). Oocyte survivability following vitrification is impacted given that the plasma membrane of oocytes is permeable to the mutual flow of both impermeable and permeable CPAs when compared to zygote and embryo (Díez et al., 2012; Hajarian et al. 2011). The highest mean percentage of 24.87% of the zygotene stage in the Cryotop treatment was significantly different (p<0.05) when compared to other oocyte groups in other treatments. However, the zygotene stage was not observed in group B' oocytes of the control group. It could result from mitochondrial dysfunction, which interferes with the assembly of the meiotic spindle and reduces ATP synthesis. The meiotic spindle is responsible for chromosomal segregation (Sasaki et al., 2019). In addition, the cellular function of bovine oocytes can be affected by several factors, such as cortical granules, cytoskeleton, and lipid droplets, which are sensitive to freezing and warming processes in cryopreservation (Prentice & Anzar, 2010).

Across the three groups of oocytes, group B' oocytes revealed the most promising outcomes in terms of the number of oocytes reaching MII. In this study, supplementation of 50 µm cysteamine/β-mercaptoethanol (antioxidants) in the IVM maturation medium for a period of 20 to 24 hr for all groups of oocytes, was expected to enhance the developmental competence of lower quality group B' oocytes, as stated by Bidin (2005). It was supported by a recent finding, which suggested the positive effect of cysteamine as an antioxidative agent in

the culture medium of IVM (Magata et al., 2021). The low-molecular-weight thiols, including cysteamine/β-mercaptoethanol, promote the formation of intracellular glutathione (GSH) in IVM media (Budani & Tiboni, 2020). *In vitro* matured oocytes have lower levels of GSH; therefore, the addition of antioxidants facilitated increased GSH levels in the cytoplasm of oocytes. GSH is important in the *in vitro* process for the maturation of oocyte cytoplasm (Nikseresht et al., 2017).

In addition, the highest mean percentage of unidentified chromosomes was recorded in group B' oocyte (27.67%) in the VS treatment compared to the control (15.26%) and Cryotop (10.77%) treatments. Despite having no oocytes in the diakinesis stage, group B' oocytes had a significantly higher ($p<0.05$) percentage of oocytes with unidentified chromosomes. Oocytes with unidentified chromosomes were higher in the VS procedure using straw compared to the Cryotop technique across all treatments. It could be due to the utilisation of a larger volume of highly concentrated solution in VS, which may have deleterious effects on the oocytes when exposed to liquid nitrogen. Besides, the technique used for inserting the samples inside the straw in the vitrification method may be detrimental to the oocytes (Rao et al., 2012). The detrimental effects during the cryopreservation procedure might elicit altered gene expression, apoptosis, and the release of oocyte-derived substances, thereby leading to cryodamage (Rao et al., 2012). Significant differences ($p<0.05$) were observed in diakinesis and MII stages compared to other meiotic stages for Cryotop treatment. Specifically, group B' oocytes recorded the lowest MII stage compared to control and VS treatments and the only treatment observed for oocytes arrested in diakinesis.

CONCLUSION

In general, fresh oocytes (control) had the highest percentage of maturation compared to the vitrification techniques. Both vitrification techniques, namely conventional VS and Cryotop, successfully maintained the maturation rate and survivability of vitrified bovine oocytes in all three groups of oocytes (A, B, and B'). In group A oocytes, a higher mean percentage was observed in the VS technique than in Cryotop. On the contrary, a higher mean percentage of MII stage in groups B and B' oocytes was observed in Cryotop compared to the conventional VS technique. These findings indicated that both vitrification techniques could be used in terms of the survival rate of bovine oocytes. Nevertheless, further studies on evaluating the effects of both vitrification techniques through IVF technique are necessary to determine the developmental competence of cryopreserved bovine oocytes.

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REFERENCES

Abd El-Aziz, A. H., Mahrous, U. E., Kamel, S. Z., & Sabek, A. A. (2016). Factors influencing *in vitro* production of bovine embryos: A review. *Asian Journal of Animal and Veterinary Advances*, *11*(12), 737–756. https://doi.org/10.3923/ajava.2016.737.756

Aguila, L., Treulen, F., Therrien, J., Felmer, R., Valdivia, M., & Smith, L. C. (2020). Oocyte selection for *in vitro* embryo production in bovine species: Noninvasive approaches for new challenges of oocyte competence. *Animals*, *10*(12), 2196. https://doi.org/10.3390/ani10122196

Aljaser, F. S. (2022). Cryopreservation methods and frontiers in the art of freezing life in animal models. In Y. Bozkurt & M. N. Bucak (Eds.), *Animal reproduction*. IntechOpen. https://doi.org/10.5772/intechopen.101750

Amidi, F., Khodabandeh, Z., & Mogahi, M. H. N. (2018). Comparison of the effects of vitrification on gene expression of mature mouse oocytes using cryotop and open-pulled straw. *International Journal of Fertility and Sterility*, *12*(1), 61–67. https://doi.org/10.22074/ijfs.2018.5112

Angel-Velez, D., De Coster, T., Azari-Dolatabad, N., Fernandez-Montoro, A., Benedetti, C., Pascottini, O. B., Woelders, H., Soom, A. V., & Smits, K. (2021). New alternative mixtures of cryoprotectants for equine immature oocyte vitrification. *Animals*, *11*(11), 3077. https://doi.org/10.3390/ani11113077

Basirat, Z., Rad, H. A., Esmailzadeh, S., Jorsaraei, S. G. A., Hajian-Tilaki, K., Pasha, H., & Ghofrani, F. (2016). Comparison of pregnancy rate between fresh embryo transfers and frozen-thawed embryo transfers following ICSI treatment. *International Journal of Reproductive Biomedicine*, *14*(1), 39–46.

Bidin, H. (2005). *IVF performance of different qualities of oocyte using co-culture and chemically-defined medium in Malaysian cattle* [Unpublished Doctoral dissertation]. Universiti Malaya.

Bidin, H., Osman, N. A., & Kamaruddin, M. (2012). Effects of oestradiol-17β on the development of cattle oocytes. In *Proceedings of the 5th International Conference on Animal Nutrition 2012 Malacca, Malaysia* (pp. 314-316). Malaysian Agricultural Research and Development Institute.

Bottrel, M., Ortiz, I., Pereira, B., Díaz-Jiménez, M., Hidalgo, M., Consuegra, C., Morató, R., Mogas, T., & Dorado, J. (2019). Cryopreservation of donkey embryos by the cryotop method: Effect of developmental stage, embryo quality, diameter and age of embryos. *Theriogenology*, *125*, 242–248. https://doi.org/10.1016/j.theriogenology.2018.11.011

Budani, M. C., & Tiboni, G. M. (2020). Effects of supplementation with natural antioxidants on oocytes and preimplantation embryos. *Antioxidants*, *9*(7), 612. https://doi.org/10.3390/antiox9070612

Cobo, A., Bellver, J., Domingo, J., Pérez, S., Crespo, J., Pellicer, A., & Remohí, J. (2008). New options in assisted reproduction technology: The Cryotop method of oocyte vitrification. *Reproductive Biomedicine Online*, *17*(1), 68–72. https://doi.org/10.1016/s1472-6483(10)60295-7

Davachi, N. D., Shahneh, A. Z., Kohram, H., Zhandi, M., Dashti, S., Shamsi, H., & Moghadam, R. (2014). *In vitro* ovine embryo production: The study of seasonal and oocyte recovery method effects. *Iranian Red Crescent Medical Journal*, *16*(9), e20749. https://doi.org/10.5812/ircmj.20749

Dhali, A., Kolte, A. P., Mishra, A., Roy, S. C., & Bhatta, R. (2018). Cryopreservation of oocytes and embryos: Current status and

opportunities. In D. S. Sheriff (Ed.), *Infertility, assisted reproductive technologies and hormone assays*. IntechOpen. https://doi.org/10.5772/intechopen.81653

Díez, C., Muñoz, M., Caamaño, J. N., & Gómez, E. (2012). Cryopreservation of the bovine oocyte: Current status and perspectives. *Reproduction in Domestic Animals*, 47(s3), 76–83. https://doi.org/10.1111/j.1439-0531.2012.02029.x

Do, V. H., Walton, S., Catt, S., & Taylor-Robinson, A. W. (2016). Requirements for cryopreservation of *in vitro*-produced bovine embryos by a standard method of vitrification. *Journal of Veterinary Science and Animal Husbandry*, *4*(1), 102. https://doi.org/10.15744/2348-9790.4.102

EXTOLSEED Oocyte and Sperm Bank (2018). *Thawing (Kitazato)*. https://extolseed.com/vitrification-and-warming-kitazato/thawing-kitazato/

Fathi, M., Moawad, A. R., & Badr, M. R. (2018). Production of blastocysts following *in vitro* maturation and fertilization of dromedary camel oocytes vitrified at the germinal vesicle stage. *PLOS One*, *13*(3), e0194602. https://doi.org/10.1371/journal.pone.0194602

Hajarian, H., Wahid, H., Rosnina, Y., Daliri, M., Dashtizad, M., Karamishabankareh, H., & Abas Mazni, O. (2011). Cryotop and development of vitrified immature bovine oocytes. *Brazilian Journal of Veterinary and Animal Sciences*, *63*(1), 67–73. https://doi.org/10.1590/s0102-09352011000100011

Hochi, S. (2022). Cryodevices developed for minimum volume cooling vitrification of bovine oocytes. *Animal Science Journal*, *93*(1), e13683. https://doi.org/10.1111/asj.13683

Idrissi, S. J., Le Bourhis, D., Lefevre, A., Emond, P., Le Berre, L., Desnoës, O., Joly, T., Buff, S., Maillard, V., Schibler, L., Salvetti, P., & Elis, S. (2021). Lipid profile of bovine grade - 1 blastocysts produced either *in vivo* or *in vitro* before and after slow freezing process. *Scientific Reports*, *11*, 11618. https://doi.org/10.1038/s41598-021-90870-8

Iussig, B., Maggiulli, R., Fabozzi, G., Bertelle, S., Vaiarelli, A., Cimadomo, D., Ubaldi, F. M., & Rienzi, L. (2019). A brief history of oocyte cryopreservation: Arguments and facts. *Acta Obstetricia et Gynecologica Scandinavica*, *98*(5), 550-558. https://doi.org/10.1111/aogs.13569

Jain, J. K., & Paulson, R. J. (2006). Oocyte cryopreservation. *Fertility and Sterility*, *86*(4), 1037–1046. https://doi.org/10.1016/j.fertnstert.2006.07.1478

Kader, A. A., Choi, A., Orief, Y., & Agarwal, A. (2009). Factors affecting the outcome of human blastocyst vitrification. *Reproductive Biology and Endocrinology*, *7*, 99. https://doi.org/10.1186/1477-7827-7-99

Keshavarzi, S., Eftekhari, A. D., Vahabzadeh, H., Mehrafza, M., Taheripanah, R., Asgharnia, M., Esfandyari, S., Ghazifard, A., Hosseinirad, H., & Paktinat, S. (2022). A comparative study of post-warming survival rates and clinical outcomes of human blastocysts vitrified/warmed by CryoTouch and Cryotop methods. *JBRA assisted Reproduction*, *26*(4), 568–573. https://doi.org/10.5935/1518-0557.20210116

Kuwayama, M. (2007). Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method. *Theriogenology*, *67*(1), 73–80. https://doi.org/10.1016/j.theriogenology.2006.09.014

Kuwayama, M., Vajta, G., Kato, O., & Leibo, S. P. (2005). Highly efficient vitrification method for cryopreservation of human oocytes. *Reproductive BioMedicine Online*, *11*(3), 300–308. https://doi.org/10.1016/s1472-6483(10)60837-1

Liu, S., & Li, F. (2020). Cryopreservation of single-sperm: Where are we today? *Reproductive Biology and Endocrinology*, *18*, 41. https://doi.org/10.1186/s12958-020-00607-x

Magata, F., Ideta, A., Matsuda, F., Urakawa, M., & Oono, Y. (2021). Glutathione ethyl ester improved the age-induced decline in the developmental competence of bovine oocytes. *Theriogenology*, *167*, 37–43. https://doi.org/10.1016/j.theriogenology.2021.03.004

Mahmoud, K. G. M., El-Sokary, M. M. M., Kandiel, M. M. M., El-Roos, M. E. A. A., & Sosa, G. M. S. (2016). Effects of cysteamine during *in vitro* maturation on viability and meiotic competence of vitrified buffalo oocytes. *Iranian Journal of Veterinary Research*, *17*(3), 165–170.

Mogas, T. (2018). Update on the vitrification of bovine oocytes and *in vitro*-produced embryos. *Reproduction, Fertility, and Development*, *31*(1), 105–117. https://doi.org/10.1071/rd18345

Nagy, Z. P., Chang, C.-C., Shapiro, D., Bernal, D. P., Kort, H. I., & Vajta, G. (2009). The efficacy and safety of human oocyte vitrification. *Seminars in Reproductive Medicine*, *27*(6), 450–455. https://doi.org/10.1055/s-0029-1241054

Nagy, Z. P., Shapiro, D., & Chang, C.-C., (2020). Vitrification of the human embryo: A more efficient and safer in vitro fertilization treatment. *Fertility and Sterility*, *113*(2), 241-247. https://doi.org/10.1016/j.fertnstert.2019.12.009

Nikseresht, M., Toori, M. A., Rahimi, H. R., Fallahzadeh, A. R., Kahshani, I. R., Hashemi, S. F., Bahrami, S., & Mahmoudi, R. (2017). Effect of antioxidants (β-mercaptoethanol and cysteamine) on assisted reproductive technology *in vitro*. *Journal of Clinical and Diagnostic Research: JCDR*, *11*(2), BC10–BC14. https://doi.org/10.7860/JCDR/2017/21778.9298

Peinado, I., Moya, I., García-Valverde, L., Francés, R., Ribes, R., Polo, P., Gómez-Torres, M. J., & Monzó, A. (2022). Potential development of vitrified immature human oocytes: influence of the culture medium and the timing of vitrification. *International Journal of Molecular Sciences*, *24*(1), 417. https://doi.org/10.3390/ijms24010417

Prentice, J. R., & Anzar, M. (2010). Cryopreservation of mammalian oocyte for conservation of animal genetics. *Veterinary Medicine International*, *2011*, 146405. https://doi.org/10.4061/2011/146405

Rao, B. S., Mahesh, Y. U., Charan, K. V., Suman, K., Sekhar, N., & Shivaji, S. (2012). Effect of vitrification on meiotic maturation and expression of genes in immature goat cumulus oocyte complexes. *Cryobiology*, *64*(3), 176–184. https://doi.org/10.1016/j.cryobiol.2012.01.005

Reyes, J. N. V., & Jaramillo, L. C. (2016). Cryopreservation method and composition of the vitrification solution affect viability of *in vitro* bovine embryos. *Revista Colombiana de Ciencias Pecuarias*, *29*, 130–137. https://doi.org/10.17533/udea.rccp.v29n2a06

Rienzi, L., Romano, S., Albricci, L., Maggiulli, R., Capalbo, A., Baroni, E., Colamaria, S., Sapienza, F., & Ubaldi, F. (2010). Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: A prospective randomized sibling-oocyte study. *Human Reproduction*, *25*(1), 66–73. https://doi.org/10.1093/humrep/dep346

Rybska, M., Knap, S., Jankowski, M., Jeseta, M., Bukowska, D., Antosik, P., Nowicki, M., Zabel, M., Kempisty, B., & Jaśkowski, J. M. (2018). Cytoplasmic and nuclear maturation of oocytes in mammals – Living in the shadow of cells developmental capability. *Medical Journal of Cell Biology*, *6*(1), 13–17. https://doi.org/10.2478/acb-2018-0003

Sanches, B. V., Zangirolamo, A. F., & Seneda, M. M. (2019). Intensive use of IVF by large-scale dairy programs. *Animal Reproduction*, *16*(3), 394–401. https://doi.org/10.21451/1984-3143-AR2019-0058

Sasaki, H., Hamatani, T., Kamijo, S., Iwai, M., Kobanawa, M., Ogawa, S., Miyado, K., & Tanaka, M. (2019). Impact of oxidative stress on age-associated decline in oocyte developmental competence. *Frontiers in Endocrinology*, *10*, 811. https://doi.org/10.3389/fendo.2019.00811

Saunders, K. M., & Parks, J. E. (1999). Effects of cryopreservation procedures on the cytology and fertilization rate of *in vitro*-matured bovine oocytes. *Biology of Reproduction*, *6*(1), 178–187. https://doi.org/10.1095/biolreprod61.1.178

Sripunya, N., Somfai, T., Inaba, Y., Nagai, T., Imai, K., & Parnpai, R. (2010). A comparison of cryotop and solid surface vitrification methods for the cryopreservation of *in vitro* matured bovine oocytes. *Journal of Reproduction and*

Development, *56*(1), 176–181. https://doi.org/10.1262/jrd.09-108h

Sydykov, B., Oldenhof, H., Sieme, H., & Wolkers, W. F. (2018). Storage stability of liposomes stored at elevated subzero temperatures in DMSO/sucrose mixtures. *PLOS One*, *13*(7), e0199867. https://doi.org/10.1371/journal.pone.0199867

Tao, T., & Del Valle, A. (2008). Human oocyte and ovarian tissue cryopreservation and its application. *Journal of Assisted Reproduction and Genetics*, *25*, 287–296. https://doi.org/10.1007/s10815-008-9236-z

Tharasanit, T., & Thuwanut, P. (2021). Oocyte cryopreservation in domestic animals and humans: Principles, techniques and updated outcomes. *Animals*, *11*(10), 2949. https://doi.org/10.3390/ani11102949

Tonev, I. D., Hristova, S. H., Zhivkov, A. M., & Mincheff, M. S. (2020). Cytotoxic effect of dimethyl sulfoxide (DMSO) on hematopoietic stem cells: Influence of the temperature and the incubation time. *Bulgarian Chemical Communications*, *52*(Special Issue B), 40–43. https://doi.org/10.34049/bcc.52.B.0011

Turathum, B., Gao, E.-M., & Chian, R.-C. (2021). The function of cumulus cells in oocyte growth and maturation and in subsequent ovulation and fertilization. *Cells*, *10*(9), 2292. https://doi.org/10.3390/cells10092292

Valdez, C. A., Abas Mazni, O., Takahashi, Y., Fujikawa, S., & Kanagawa, H. (1992). Successful cryopreservation of mouse blastocysts using a new vitrification solution. *Journal of Reproduction and Fertility*, *96*(2), 793–802. https://doi.org/10.1530/jrf.0.0960793

Vining, L. M., Zak, L. J., Harvey, S. C., & Harvey, K. E. (2021). The role of apoptosis in cryopreserved animal oocytes and embryos. *Theriogenology*, *173*, 93–101. https://doi.org/10.1016/j.theriogenology.2021.07.017

Vladimirov, I. K., Tacheva, D., & Dobrinov, V. (2019). The present and future of embryo cryopreservation. In B. Wu & H. L. Feng (Eds.), *Embryology: Theory and practice*. IntechOpen. https://doi.org/10.5772/intechopen.80587

Whaley, D., Damyar, K., Witek, R. P., Mendoza, A., Alexander, M., & Lakey, J. R. T. (2021). Cryopreservation: An overview of principles and cell-specific considerations. *Cell Transplantation*, *30*, 1-12. https://doi.org/10.1177/0963689721999617